

Figures 4 A-C show the effect of the AHM method on cell proliferation; A. shows how anti-sense bFGF sensitizes HeLa cells to Fas induced PCD; B. shows the levels of expression of bFGF; C. shows the quantitation of the levels bFGF forms;

Figures 5 A-D show the effect of the AHM method on cell proliferation; A. shows how anti-sense Nrf2 sensitizes HeLa cells to Fas induced PCD; B. shows the levels of expression of Nrf2; C. shows how Dicumarol sensitizes HeLa cells to Fas induced PCD; D. shows how N-acetyl cysteine protects HeLa cells from Fas induced PCD; and

Figures 6 A-C show the effect of the AHM method; A. shows "Function Profiling" generated by cDNA microarray analysis; B. shows the distribution of the differential abundance of cDNAs contained in the microarray; C. shows "Sensitizing" cDNAs candidates identified by microarray analysis. F

Page 17, line 19, after “1990.], please insert the following new paragraphs:

Also disclosed by the present invention is a method for the identification of genes that encode for inhibitors of cell death. This method is commonly known as the achilles heel method (AHM). This method involves introducing an antisense library into a vector such as an episomal vector (Deiss and Kimchi, 1991) enters target cells to generate a pool of cells with each cell expressing a different antisense fragment. This pool of cells will be known as Pool 1. Second, the transfectants are treated with a sub-optimal dose of an

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inducer and the surviving cells are collected. The surviving cells are known as Pool 2. The cells containing inactivation events that sensitize the cells to death are preferentially lost from Pool 2, and so are the antisense CDNA inserts that confer the sensitization. These CDNA inserts are recovered by subtracting the CDNA inserts containing in Pool 2 from those in Pool 1. The products of the subtraction are cloned in the episomal expression vector and individually transfected into target cells in order to confirm their ability to render the cells more sensitive to the killing inducer.

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Following the subtraction of Pool 2 cDNAs from Pool 1 cDNAs, the potentially sensitizing cDNAs are cloned in an anti-sense orientation in an episomal expression vector. The anti-sense cDNA containing episomes are individually transfected into target cells in order to confirm their ability to render the cells more sensitive to the killing inducer. Alternatively, Pool 1 and Pool 2 cDNAs are labeled and used as probes for hybridization of cDNA microarray filter. Computer analysis identifies the cDNAs depleted from Pool 2. In both cases "function profiling" is being employed to identify signal pathway inhibitors. Recently, similar "function profiling" methods have been described for genetic analysis of S.cerevisiae by .. et al (Pat Brown and Ron Davis). These methods are well suited to yeast since they require prior knowledge of gene sequence and the ability to generate haploid cells. By contrast, AHM does not require a priori knowledge of any gene sequence or haploid cells. Thus, AHM is a powerful genetic tool for "function profiling" in mammalian cells. Moreover, AHM can be easily scaled up to generate "function profiles" of all expressed human genes.

The AHM and the TKO methods are complimentary and together can be used to identify the positive and negative regulators of any pathway that can be reconstructed into human cells and culture. The AHM method identifies genes whose inactivation by antisense sensitize cells to an inducer. Thus, it

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Conversely, the inhibition of Fas activated killing can also have clinical benefit under certain circumstances. For example the inhibition of Fas induced killing can protect the liver from acute damage. The inhibition of the Fas pathway by over expression of the gene identified in the AHM Fas screen inhibits Fas induced cell killing. These genes are secreted molecules, thus the addition of the soluble molecule protect cells from damage. Over expression of the inhibitors can also be achieved through a number of other means including the use of gene therapy to transduce the gene into potential target cells and protect those cells.

There are other uses of the AHM method that are directly related to the validation of AHM in identifying inhibitors of the Fas pathway. One application is to identify toxic drug interactions. Performing an AHM screen to identify genes that inhibit toxic side effects of a specific drug (drug X) is one example. It is important that the genes that are identified as inhibitors of drug X induced toxicity be fully functioning. When one of the identified genes is found to be inhibited by another drug (drug Y) then that drug Y sensitizes patients to drug X. This type of analysis permits rapid screening for drug interactions before the drugs were used on patients.

The genes identified in the Fas AHM screen are survival factors since they inhibit killing induced by Fas. As such they can be used to limit chronic or acute pathological situations in which there is excessive cell death. Such applications could include organ failure induced by heart failure, acute liver damage and ischemic stress.

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Additionally, the identified targets can then be used as targets to develop inhibitors and those inhibitors can be used as cofactors to activate the identified pathway.

For example, one can identify *genes*, which inhibit killing induced by chemotherapeutics. Inhibition of such genes sensitizes tumors to chemotherapeutics. Such inhibitors have utility in treating cancer patients.

A list of genes were identified in the Fas AHM screen using the gene blot analysis. These genes are identified as inhibitors of Fas induced killing and are shown in Table 3.

Page 44, line 14, after "selected phenotype." please insert the following paragraphs:

EXAMPLE 3:

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The Achilles Heel Method utilizes functional profiling as diagrammed in Figure 3. The first step consists of introducing an anti-sense expression library (Deiss and Kimchi, 1991) into target cells to generate a pool of cells, each expressing a different anti-sense fragment (Pool 1). Then, the transfectants are treated with a sub-optimal dose of a PCD inducer and the surviving cells are collected (Pool 2). Cells containing inactivation events that sensitize the cells to killing are preferentially lost from Pool 2. Consequently, the anti-sense cDNAs contained in the sensitized cells are depleted from Pool 2. The "sensitizing" cDNA inserts that are present in Pool 1 but depleted from Pool 2 are identified by two methods, subtraction or hybridization to cDNA microarray. Following the subtraction of Pool 2 cDNAs from Pool 1 cDNAs, the potentially sensitizing cDNAs are cloned in an anti-sense orientation in an episomal expression vector. The anti-sense cDNA containing episomes are

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For the identification of inhibitors of Fas induced cell death, AHM was applied to HeLa cells that were treated with sub-lethal dose of Fas agonistic antibody. The later mimics the binding of Fas ligand to Fas and induces apoptosis. "Function profiling" was performed to identify "sensitizing" cDNA fragments by using subtraction and gene array analysis. cDNA inserts from Pool 2 were subtracted from Pool 1 cDNAs and the recovered cDNAs were further analyzed. Sequencing of 226 fragments revealed 168 unique sequences, of which 53% are novel and 47% correspond to known genes. Six out of seven randomly chosen cDNAs that were individually transfected into HeLa cells conferred increased sensitivity to Fas induced killing cells, ranging between 2.9 to 5.3 fold. These fragments include three novel sequences and three fragments of previously described genes. One of the cDNA inserts is an anti-sense fragment of human Basic Fibroblast Growth Factor (FGF-2, bFGF) and the other is an anti-sense fragment of the cap-n-collar b-zip transcription factor NF-E2 related factor 2 (NRF2).

bFGF is a potent survival factor that plays a role in development, angiogenesis and in cell migration. Previous reports show that down regulation of bFGF by anti-sense expression or by blocking antibodies result in loss of a transform phenotype, reduced tumor growth and reduced angiogenesis. Five different polypeptides of 34kD, 24kD, 22.5kD, 22kD and 18 kD are translated from the human bFGF gene, initiating at different sites and terminating at the same position. The anti-sense cDNA fragment isolated in the subtraction is 295 nucleotides long and corresponds to nucleotide 890 to 1184 of the bFGF gene. It spans the last 60 nucleotides of the coding region (shared by all bFGF polypeptides) and a portion of the 3' untranslated region.

In order to confirm that anti-sense bFGF confers sensitivity to Fas, pools of cells transfected with control vector (harboring no insert) or with anti-sense bFGF were generated and treated with sub-optimal dose of anti-Fas

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Western blot analysis of control vector transfected cells as well as anti-sense bFGF transfected cells revealed four polypeptides of 24kD, 22/22.5kD and 18kD, while the 34kD form is not detected by the antibody used (Figure 4B). Quantitative analysis of the relative level of bFGF forms revealed that in the absence of anti-Fas antibody, expression of anti-sense bFGF results in reduction of approximately 25%-30% in the levels of each of the detected forms (Figure 4C). Interestingly, in cells treated with anti-Fas antibody a more significant reduction in the levels the 24kD form and 18kD is observed, 57% and 66% respectively, while the reduction of the levels of the 22/22.5kD is not altered. Selective reduction in the level of some of the bFGF forms by an anti-sense fragment that overlaps the coding region of all the bFGF polypeptides can be due to a network of feedback regulation loops as previously reported for some bFGF forms.

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While previous studies has shown that over-expression of the 34kD form protects cells from serum deprivation induced killing and over-expression of the 24kD form protects cells from ionizing radiation, here it is demonstrated that bFGF is an inhibitor of Fas induced apoptosis, as identified by AHM.

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The second inhibitor of the Fas pathway that was identified by AHM is the cap-n-collar b-zip transcription factor NF-E2 related factor 2 (Nrf2). Nrf2 activates the transcription of phase II detoxifying enzymes such as NAD(P)H quinone oxireductase (NQO1) and Glutathione S-transferase (GST) by direct binding to the Antioxidant Response Element (ARE) in the promoter of these genes. Studies of nNrf2 null mice indicate that Nrf2 is essential for the transcriptional activation of phase II enzymes. NQO1 and GST act in concert with phase I detoxifying enzymes (such as cytochrome p-450 monooxygenase) to mediate the cellular detoxification of xenobiotics. In the absence of Nrf2, this coordinated detoxification is impaired and toxic products from phase I reactions can accumulate. In the AHM screen an anti-sense fragment of Nrf2 corresponding to nucleotide 147 to 970 of the human Nrf2 (gbS74017, Moi et al, 1994, PNAS 21, 9926) was recovered.

Bioassays of two pools of HeLa cells transfected with anti-sense Nrf2 clearly demonstrates that anti-sense Nrf2 render the cells 4.1 and 5.4 fold more sensitive to Fas induced apoptosis (Figure 5A). Again, this increase sensitivity is not a result of impaired growth, since there is only limited alteration in the growth rate of anti-sense Nrf2 transfected cells (Figure 5A). Sensitization by anti-sense Nrf2 was reproducible in seven independent pools of transfectants. Western blot analysis indicated a significant 3.8 fold reduction in the level of Nrf2 protein in the anti-sense Nrf2 transfected cells (Figure 5B).

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A technically simpler alternative to "function profiling" by subtraction is analysis of cDNA microarray. The relative abundance of cDNAs was measured in Pool 1 and in Pool 2 by radio-labeling each pool and hybridizing each of the probes to a cDNA microarray containing approximately 4,000 different known human genes. Figure 6A is a pseudo colored image of

the microarray filter representing for each cDNA spot the ratio of signal generated by hybridization to Pool 2 probe to that generated by Pool 1 probe. Dark green spots indicate cDNAs absent in Pool 2, representing "sensitizing" anti-sense cDNAs. The corresponding genes are predicted to be survival factors that inhibit Fas induced apoptosis. Dark red spots indicate cDNAs that are enriched in Pool 2. These genes are positive mediators of killing and their inactivation by anti-sense results in resistance to PCD. The abundance of such anti-sense cDNAs is therefore increased in Pool 2 that is comprised of cells that survived Fas induced apoptosis. Most of the spots are of intermediate color indicating only modest changes in abundance in Pool 2 relative to Pool 1. A histogram representation of the results is shown in Figure 6B. As seen, the abundance of the majority of cDNAs is not changed. However, a small number of cDNAs are depleted from Pool 1 by 2 folds or more. A partial list of these genes is presented in Figure 6C. As predicated, these genes include survival factors. For example, the most depleted cDNA (5.6-fold) corresponds to TNF receptor associated factor 6 that relays a strong survival signal via the activation of NFkB and AKT. In addition casein kinase 1 alpha and adenosine A3 receptor have been shown to be survival factors.

In summary, this is a novel powerful tool for identifying signaling inhibitors in human cells. Thus, a large gap in the genetic analyses of mammalian cells has now been filled. AHM can be broadly used to identify inhibitors of any given selectable pathway for the purposes of basic research or clinical applications. Moreover, since it does not require previous knowledge of any sequences, AHM can be employed as a high throughput method of gene discovery and "function profiling" as part of the ongoing effort of deciphering the human genome.

Materials and Methods:

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AHM: HeLa cells (10^6 cells/100 mm plate) were transfected with 15 ug of anti-sense cDNA library in pTKO-1 (Deiss and Kimchi 1991) by Superfect reagent (Qiagen). Two days later cells were treated with 200 ug/ml Hygromycin B (Calbiochem-Novabiochem) for two weeks. 2.5×10^6 Hygromycin^R cells were plated in a 150 mm plate 24 hours prior to treatment with 10 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company) (Pool 2). Five days post treatment approximately 30-40% of the cells were killed as estimated by microscopic examination. A parallel culture was grown in the absence of anti-Fas antibody (Pool 1). After five days, cells were washed twice with PBS, scraped off the plate and stored as pellets at -80° . 100 ul of frozen pellet were lysed by addition of 200 ul of solution P1, followed by 200 ul of solution P2. After the lysate sat on ice for 5 minutes 200 ul of solution P3 was added (Qiagen plasmid purification kit). Following 5 minutes incubation on ice, the lysate was centrifuged for 10 minutes at $15,000 \times g$, the supernatant was mixed with an equal volume of isopropanol and centrifuged at $15,000 \times g$ for 10 minutes. The DNA pellet was rinsed with 70% ethanol and resuspended in 100 ul of water. The cDNA inserts were amplified by PCR in a 100 ul reaction containing: 1 ul DNA, 200 uM of dATP, dGTP, dCTP, dTTP; 10 mM Tris-HCl pH9.0; 0.1 % Triton X-100; 1.0mM MgCl; 1 unit Taq DNA polymerase (Gibco BRL) and 500 ng each of primers: prLPD#64 (TGGAGGCCTAGGCTTTTGC) and prLPD#65 (GTAAGGTTCTTCACAAGGATCC). These primers are derived from the sequences that flank the cDNA insertion site in the pTKO-1 anti-sense expression vector. The primers are designed to restore a HindIII restriction site on the promoter proximal side of the cDNA and a BamHI site on the promoter distal side to conserve the orientation of the cDNA fragments upon their cloning in pTKO-1. The reaction was incubated 94°C for 5 minutes; subjected to 25 cycles of: 94°C for one minute, 58°C for one minute and 72°C for five minutes; followed by 72°C for seven minutes. The PCR products were cleaved by BamHI and HindIII, purified (Wizard PCR Prep Kit, Promega) and used in subtraction (PCR-Select kit, Clontech). The driver for the subtraction

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was the product of the PCR reaction derived from the untreated cells (Pool 1) and the tester was derived from treated cells (Pool 2). The following modifications to the manufacturer's instructions were made: 1. The first step was IV F 3, since no cDNA synthesis is required. 2. The blunt ends adapters 1 and 2R were replaced with cohesive ends adapters as follows: Adapter 1 was replaced by a mixture of primers prLPD#80 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGCAGGTA), prLPD#81 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGCAGGTG), prLPD#83 (AGCTTACCTGCCCCGG) and prLPD#84 (GATCCACCTGCCCCGG). Adapter 2R was replaced by a mixture of prLPD#82 (CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGTG), prLPD#88 (AGCTTACCTCGGCCG), prLPD#89 (GATCCACCTCGGCCG) and prLPD#90 (CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGTA).

Cohesive end adapters ligate more efficiently to the cDNA and permit the directional cloning of the cDNA inserts. 0.3 ug of the tester was used for adapter ligation. 3. The initial hybridization included 0.9 ug of the driver and 0.03 ug of the adapted ligated tester. The products of the subtraction were cleaved with BamHI and HindIII, purified and cloned into the pTKO-1 between BglII and HindIII sites. Individual clones were sequenced and transfected into HeLa cells.

Transfection and Bioassays: HeLa cells (2×10^6 cells/100 mm plate) were plated 20 hours prior to transfection with either 17 ug of either anti-sense expressing vector or control vector harboring no cDNA insert, by calcium phosphate. Forty eight hours post transfection cells were treated with 200 ug/ml Hygromycin B (Calbiochem-Novabiochem) for two weeks. For bioassays, anti-sense transfected cells or control vector transfected cells (1.6×10^5 cells/

well in 6 wells plates) were plated 20-24 hours prior to the treatment with 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 24 hours post treatment

Western analysis: Anti-sense transfected cells or control vector transfected cells (2.5×10^6 cells/150mm plate) were plated 24 hours prior to treatment with 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). 24 hours post treatment cells were washed with PBS and lysed in RIPA buffer (1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 2mg/ml aprotonin and 2mg/ml pepstatin in PBS). Samples containing 50mg protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The immunoblots were probed with either anti-Nrf2 antibody (1:100, Santa Cruz, sc722) or anti-bFGF-2 antibody, (1:200, Santa Cruz, sc 079), incubated with goat anti rabbit conjugated to horseradish peroxidase (Pierce) followed by incubation with SuperSignal substrate (Pierce). Following autoradiography, the probes were stripped off (Amersham, ECL Western blotting protocols) and the membranes were hybridized with anti-actin antibody, (1:100, Sigma A4700 or A2066). The intensities of the bands were quantified by the National Institute of Health Image program.

Treatment with N-acetyl cysteine: HeLa cells (8.3×10^4 cells /well in 6 wells plates) were plated 20-24 hours prior to treatment with various concentrations of NAC (Sigma/Aldrich,) in the presence or absence of 50 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 5 days post treatment.

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Treatment with Dicumarol: HeLa cells, 1.6×10^5 cells /well were plated in 6 wells plates. 20-24 hours later cells were treated with various concentrations of dicumarol (Sigma/Aldrich,) in 0.2 mM NaOH for 15 minutes prior to the addition of 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 17 hours post treatment.

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cDNA microarray analysis: Approximately 500 ng of the PCR products of Pool 1 and Pool 2 (same preparations that were used for the subtraction, before their cleavage by BamHI and HindIII) were labeled with 100 mCi of [^{33}P] dCTP (3000 Ci/mmol, ICN,) by the random primers DNA labeling system (Gibco/BRL), purified (Aershm/Pharmacia, ProbeQuant G50 micro columns) and individually hybridized to Human GeneFilters (GF211, Research Genetics). The filter was pre-hybridized for 40-60 minutes at 68 °C in ExpressHyb Hybridization solution (Clontech), followed by hybridization for 3-5 hours at 68°C. The filter was washed in 2xSSC, 0.05% SDS at room temperature 3-5 times for 10-15 minutes each time followed by 2 washes for 15 minutes each in 0.1x SSC, 0.1% SDS at 55°C. The image was generated by Molecular Dynamics phospho-imager. In between hybridizations, the probe was stripped off by adding boiling solution of 0.5% SDS and incubating at room temperature for 1 hour. Successful removal of probe was confirmed by phosphor-imager analysis. Images processing and calculation of the ratio of the signals of Pool 2 probe to Pool 1 probe were performed by Pathways II software (Research Genetics). All the spots that showed significant differential abundance were visually inspected.

Page 51, after "Table 2", please insert the following Table:

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